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# Changes in Lipid Composition After In-Vitro Selection for Glyphosate Tolerance in Tobacco

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**Abstract:** After a one-step selection procedure with glyphosate added to the callus medium, tobacco regenerants were obtained from calluses surviving on field doses of the herbicide. The lipid and sterol compositions of potted plants from the two original cultivars which underwent the selection procedure but without glyphosate, and the selected regenerants were investigated. Controls were derived from micropropagation and not from seeds. They served as appropriate controls for comparison with glyphosate regenerants, i.e. they underwent exactly the same experimental conditions except for the glyphosate treatment.

Plant regeneration was strongly (at one-step selection) or fully (at stepwise selection) inhibited. Only a few glyphosate-tolerant plants (seven of *Nevrokop* $A_{24}$ and five of *Zlatna arda*) were obtained which were cloned and potted. At least 10 plants of one clone per genotype were used for the further biochemical analyses.

The spraying of the tobacco plants from the cultivars with glyphosate led to a decrease of monogalactosyl diacylglycerol content and increase of the saturated fatty acids in all glycolipid classes. The concentration of stigmasterol increased and that of sitosterol decreased only in *Zl.arda* variety.

In the regenerants there were different changes in lipid composition, concerning mainly a decrease of monogalactosyl diacylglycerols (MGDG) and an increase of digalactosyl diacylglycerols (DGDG) in *Zl.arda*-derived regenerants and increase of the amounts of neutral lipids and decrease of phospholipids (PL) in the *Nevrokop* $A_{24}$-derived regenerants. Surprisingly, after treatment of the regenerants with glyphosate, the MGDG amounts in these regenerants were higher than in the controls, which could mean a lesser ability to control ion permeability.

Key words: glyphosate, tobacco, lipids, sterols.

## 1 INTRODUCTION

Glyphosate (*N*-(phosphonomethyl)glycine) is a broad-spectrum, post-emergence herbicide with a number of valuable properties. It is applied extensively throughout the world in many different situations.[1]

The primary site of action of the herbicide is the shikimate pathway, where it acts as a competitive inhibitor of enol pyruvylshikimate phosphate synthase.[2–4] Since this aromatic amino acid biosynthetic pathway is common to all plants, glyphosate-tolerant plant forms must be created by artificial means. Their availability will make possible the application of this total herbicide in a selective manner. Genetic engineering methods can offer an efficient and relatively fast solution to this problem. However, cell selection procedures are acceptable alternatives in comparison with the more sophisticated and expensive methods of somatic hybridization or gene cloning and transfer methods.

It is known that the lipid and sterol composition of plants may vary with environmental conditions and

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these changes are connected with the structure and functions of the lipid cell membrane and may be of adaptive value or may be attributed to stress-induced degradation processes. No data are available on the changes in lipid and sterol composition of plants caused by treatment with glyphosate.

The aim of our study was to select tobacco cell lines tolerant to field doses of glyphosate, to regenerate plants expressing this important trait and to study the changes in the lipid and sterol composition in the plants and regenerants before and after treatment with glyphosate, which could be of adaptive value.

## 2 EXPERIMENTAL

### 2.1 Plant material

For callus induction, aseptically maintained seedlings of the oriental-type Bulgarian tobacco (*Nicotiana tabacum* L.) cultivars *Zlatna arda* and *Nevrokop* $A_{24}$ were used. These genotypes are widely cultivated in Bulgaria. The in-vitro procedure developed included micropropagation and maintaining by node cuttings on hormone-free MS medium.[5]

The selection procedure was described earlier.[6] Briefly it includes in-vitro germination of seeds and cloning of seedlings from two oriental type Bulgarian tobacco cultivars (*Zlatna arda* and *Nevrokop* $A_{24}$) on basal MS medium,[5] followed by callus induction from stem cuttings on MS medium supplemented with casein hydrolysate (500 mg litre$^{-1}$), NAA (1 mg litre$^{-1}$) and kinetin (0·5 mg litre$^{-1}$). The regeneration was carried out on MS with casein hydrolysate (250 mg litre$^{-1}$), IAA (0·2 mg litre$^{-1}$), kinetin (0·5 mg litre$^{-1}$) for the first stage and on MS with casein hydrolysate (500 mg litre$^{-1}$), IAA (0·2 mg litre$^{-1}$), kinetin (1 mg litre$^{-1}$) and $GA_3$ (0·05 mg litre$^{-1}$) for the second stage.

Two different selection procedures were followed: *one-step*, with glyphosate (acidic form of glyphosate, >90%, supplied by the Monsanto Office in Bulgaria) addition to the callus medium and regeneration on herbicide-free media, and *stepwise*, with herbicide present in callus and regeneration media.

The herbicide was filter-sterilized and added to the media at 5, 10, 20, 40, 60 or 80 mM doses.

The in-vitro experiments were carried out three times with at least 10 tubes per herbicide concentration.

Regenerants were obtained only after the one-step selection procedure. They were then cloned and transferred to soil under greenhouse conditions.

Micropropagated seedlings from both cultivars were used as controls during the whole procedure. When the potted plants (regenerants and controls) reached the seven- to eight-leaf stage, half of them (10–15 per genotype) were sprayed with 20 mM (field dose) of glyphosate solution. Four days after the treatment, all leaves of the sprayed and unsprayed plants were collected for further biochemical tests.

### 2.2 Quantitative determination of the main lipid classes and their fatty acid composition

The fresh plant leaves, collected from at least 10 plants, were homogenized immediately after collection with chloroform + methanol (1 + 1 by volume) and refluxed for a few minutes in order to inactivate the enzymes. The extraction of the samples and the purification of the extracts was performed according to the method of Bligh & Dyer.[7]

A portion of the total lipid extract was separated by preparative TLC (silica gel G) with chloroform + methanol + acetone + acetic acid (35 + 7 + 12 + 0·2 by volume). The spots of the main lipid classes (NL, MGDG, DGDG and PL) were located under UV light after spraying with fluorescent indicator[8] and then scraped off. The lipid spots were identified by their chromatographic behaviour, compared to that of authentic samples, and by specific spray reagents: Dittmer and Lester spray for PL, and alpha-naphthol for GL.[9] After the addition of internal standard (heptadecanoic acid) the different lipid groups were trans-esterified with methanol + acetyl chloride, according to Christie.[9] Analysis of the FAME was carried out by FID–GLC: glass capillary column (30 m × 0·2 mm ID), coated with 'Silar' 10C. In order to determine the amounts of each lipid class, the weights of FAME were multiplied by a *K*-factor.[10] The conversion factors used were $K = 1{\cdot}05$ for NL, $K = 1{\cdot}43$ for MGDG, $K = 1{\cdot}77$ for DGDG and $K = 1{\cdot}38$ for PL.

### 2.3 Sterol analysis

A portion of the total lipids was hydrolyzed by refluxing in 1 M ethanolic potassium hydroxide for 2 h. Unsaponified compounds were extracted with diethyl ether and sterols isolated by preparative TLC on silica gel G (hexane + diethyl ether, 1 + 1 by volume). The recovered sterols were analyzed by FID–GLC (glass capillary column 30 m × 0·2 mm ID, OV-17) at 260° and by MS (70 eV).

## 3 RESULTS AND DISCUSSION

### 3.1 Plant experiments

Controls were derived from micropropagation and not from seeds. They served as appropriate controls for comparison with glyphosate regenerants, i.e. they

**TABLE 1**
Influence of Glyphosate on the Composition of the Main Lipid Classes in Leaves of the Tobacco Cultivar *Zlatna arda*

| Sample | Lipid content | |
|---|---|---|
| | *mg g⁻¹ dry wt (±SD)*[a] | *% of total* |
| Control, unsprayed | | |
| NL | 14·8 (±0·9) | 29·6 |
| MGDG | 9·2 (±0·9) | 18·4 |
| DGDG | 17·6 (±1·0) | 35·2 |
| PL | 8·4 (±0·7) | 16·8 |
| Control, sprayed | | |
| NL | 20·7 (±1·0) | 42·4 |
| MGDG | 6·9 (±0·5) | 14·1 |
| DGDG | 12·3 (±0·9) | 25·1 |
| PL | 9·0 (±0·8) | 18·4 |
| Glyphosate-tolerant regenerant, unsprayed | | |
| NL | 12·6 (±0·8) | 25·8 |
| MGDG | 2·7 (±0·3) | 5·5 |
| DGDG | 24·0 (±1·1) | 49·1 |
| PL | 9·6 (±0·8) | 19·6 |
| Glyphosate-tolerant regenerant, sprayed | | |
| NL | 7·5 (±0·6) | 14·6 |
| MGDG | 8·0 (±0·7) | 15·5 |
| DGDG | 19·5 (±1·0) | 37·9 |
| PL | 16·5 (±1·0) | 32·0 |

[a] $n = 3$.

**TABLE 2**
Influence of Glyphosate on the Composition of the Main Lipid Classes in Leaves of the Tobacco Cultivar *Nevrokop* $A_{24}$

| Sample | Lipid content | |
|---|---|---|
| | *mg g⁻¹ dry wt (±SD)*[a] | *% of total* |
| Control, unsprayed | | |
| NL | 13·0 (±0·9) | 25·0 |
| MGDG | 5·0 (±0·4) | 9·6 |
| DGDG | 12·5 (±0·9) | 24·0 |
| PL | 21·5 (±0·7) | 41·4 |
| Control, sprayed | | |
| NL | 7·6 (±0·6) | 14·0 |
| MGDG | 4·4 (±0·4) | 8·1 |
| DGDG | 22·0 (±1·1) | 40·4 |
| PL | 20·4 (±0·8) | 37·5 |
| Glyphosate-tolerant regenerant, unsprayed | | |
| NL | 22·8 (±1·0) | 43·5 |
| MGDG | 6·8 (±0·5) | 13·0 |
| DGDG | 12·8 (±0·9) | 24·4 |
| PL | 10·0 (±0·9) | 19·1 |
| Glyphosate-tolerant regenerant, sprayed | | |
| NL | 15·2 (±0·9) | 26·4 |
| MGDG | 7·8 (±0·6) | 13·5 |
| DGDG | 28·0 (±1·0) | 48·6 |
| PL | 6·6 (±0·5) | 11·5 |

[a] $n = 3$.

underwent exactly the same experimental conditions, except the glyphosate treatment.

Significant differences were found between the Growth Index (GI) values of callus cultures maintained on control and herbicide-containing callus medium in the one-step selection scheme.[6] The callus growth was strongly (three to six times) inhibited in the presence of herbicide. There were small and insignificant variations in the GI values at the herbicide concentrations used. The growth of the *Zlatna arda* cultures was strongly suppressed even at 5 mM glyphosate in the callus medium, while comparable GI values for the *Nevrokop* $A_{24}$ were reached only at 60 mM of herbicide.[6] This is an indication of the greater inherent tolerance of *Nevrokop* $A_{24}$ towards glyphosate.

When the presence of glyphosate was continued in the regeneration media (stepwise selection procedure) the callus culture growth was fully inhibited even at herbicide concentration of 5 mM.

Plant regeneration was strongly (at one-step selection) or fully (at stepwise selection) inhibited. Only a few glyphosate-tolerant plants (seven of *Nevrokop* $A_{24}$ and five of *Zlatna arda*) were obtained which were cloned and potted. At least 10 plants of one clone per genotype were used for further biochemical analyses.[6]

### 3.2 Lipid and sterol composition of leaves of both tobacco cultivars

The lipid composition of plants from the two cultivars tested differed substantially (Tables 1 and 2). While the relative concentrations of neutral lipids (NL) were similar, in *Nevrokop* $A_{24}$ the glycolipid (GL) concentration was lower. Contrary to most plants the monogalactosyl diacylglycerols (MGDG) concentrations in both cultivars are lower than these of digalactosyl diacylglycerols (DGDG).

The fatty acid (FA) composition of both tobacco cultivars was similar to that found in other 18 : 3-type higher plants, the main fatty acids being palmitic, stearic, linoleic and linolenic, accompanied by 15 : 0, 16 : 1, 17 : 1 and 18 : 1 acids in lower concentrations (Table 3 and 4). Saturated acids appeared in higher concentrations in cultivar *Zl.arda*, especially in phospholipids (PL), while in *Nevrokop* $A_{24}$ unsaturated acids predominated, mainly in PL. It could be speculated that lipid cell membranes of *Nevrokop* $A_{24}$ are more fluid, but it is more likely that this is a compensation for the increased concentration of PL in them. Also, the 50% lower concentration of MGDG in *Nevrokop* $A_{24}$ could

**TABLE 3**
Influence of Glyphosate on the Fatty Acid Composition of Main Lipid Classes in Leaves of the Tobacco Cultivar *Zlatna arda*

| Sample | *Fatty acids (% w/w)*[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | *< 16 : 0* | *16 : 0* | *16 : 1* | *16 : 2* | *18 : 0* | *18 : 1* | *18 : 2* | *18 : 3* |
| Control, unsprayed | | | | | | | | |
| NL | 5·7 | 32·4 | — | 6·6 | 7·6 | 7·6 | 17·0 | 23·1 |
| MGDG | 22·8 | 30·2 | — | — | 7·9 | 7·8 | 9·5 | 21·8 |
| DGDG | 7·6 | 42·6 | 3·4 | — | 10·0 | 7·3 | 9·7 | 19·4 |
| PL | 5·7 | 59·8 | 5·6 | — | 11·7 | 6·5 | 3·2 | 7·5 |
| Control, sprayed | | | | | | | | |
| NL | — | 33·8 | 3·3 | — | 6·7 | 14·1 | 18·0 | 24·1 |
| MGDG | 33·5 | 38·3 | — | — | 11·3 | 9·1 | 4·6 | 3·2 |
| DGDG | 23·1 | 56·0 | — | — | 15·8 | 3·9 | 1·2 | tr |
| PL | 8·3 | 64·4 | 4·7 | | 10·2 | 9·3 | 3·1 | tr |
| Glyphosate-tolerant regenerant, unsprayed | | | | | | | | |
| NL | — | 22·2 | — | — | 5·5 | 5·5 | 27·4 | 39·4 |
| MGDG | 20·9 | 30·5 | — | — | 3·9 | 6·2 | 14·2 | 24·3 |
| DGDG | | 20·1 | — | — | 5·0 | 7·9 | 18·5 | 48·5 |
| PL | — | 31·0 | 2·5 | — | 4·2 | 5·2 | 26·3 | 30·8 |
| Glyphosate-tolerant regenerant, sprayed | | | | | | | | |
| NL | 4·7 | 23·6 | — | — | 5·7 | 10·0 | 17·8 | 38·2 |
| MGDG | 34·0 | 38·4 | — | — | 8·2 | 11·7 | 4·9 | 2·8 |
| DGDG | 19·3 | 58·9 | 2·6 | — | 13·0 | 4·2 | 2·0 | — |
| PL | — | 39·2 | — | — | 3·2 | 20·4 | 28·7 | 8·5 |

[a] Values obtained from three parallel measurements. The standard deviations (related to peak proportion on the chromatograms) are as follows: $\pm 0{\cdot}2$ for $C_{<16:0}$, $C_{16:1}$ and $C_{18:0}$; $\pm 0{\cdot}3$ for $C_{18:1}$; $\pm 0{\cdot}5$ for $C_{18:2}$ and $C_{16:0}$; $\pm 0{\cdot}6$ for $C_{18:3}$.

compensate for the increased concentration of unsaturated FA. It is known[11] that, because of its hexagonal (2)-structure, a reduced level of MGDG may indicate a higher control of ionic permeability in the membrane.

The substantial differences found in the lipid compositions of both cultivars may lead to differences in the functions of their lipid cell membranes and eventually to different tolerances toward glyphosate.

The cultivars had almost identical sterol compositions, common for higher plants (Table 5).

### 3.3 Changes in lipid and sterol composition of tobacco cultivars, caused by treatment of the plants with glyphosate

The treatment of *Zl.arda* plants caused a decrease in the MGDG and DGDG concentrations and increase of PL and especially of NL concentrations (Table 1). The changes in *Nevrokop* $A_{24}$ were opposite to those seen in *Zl.arda* (Table 2). The only similarity between the two cultivars after the herbicide treatment was the small decrease of the MGDG amounts in both samples. This was in accordance with the above-mentioned increase of the control of ionic permeability of membranes, caused by the decrease in MGDG content. It is possible that the first reaction of plants after glyphosate treatment is to reduce membrane permeability and this can easily be obtained by a reduction of MGDG concentrations.

In both cultivars the concentration of saturated FA in GL increased, which was in agreement with our expectations that chloroplasts, whose membranes contain mainly GL, will sustain larger changes after glyphosate treatment. There was also a large increase of the concentration of the rare 15 : 0 acid. In PL of *Zl.arda* the concentration of linolenic acid decreased substantially after treatment with glyphosate.

The treatment with glyphosate caused significant changes in the sterol composition only in *Zl.arda* (Table 5). The concentration of stigmasterol increased and that of sitosterol decreased after the treatment. It is known that these changes lead to a decrease of cell membrane permeability[12] analogously to the changes of the lipid composition discussed above.

### 3.4 Changes in lipid and sterol composition of tobacco regenerants from calluses, maintained on glyphosate

There were different changes in the lipid (Tables 1 and 2) and sterol (Table 5) composition of the regenerants

**TABLE 4**
Influence of Glyphosate on the Fatty Acid Composition of Main Lipid Classes in Leaves of the Tobacco Cultivar *Nevrokop* $A_{24}$

| | *Fatty acids (% w/w)*[a] | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Sample* | *<16 : 0* | *16 : 0* | *16 : 1* | *16 : 2* | *18 : 0* | *18 : 1* | *18 : 2* | *18 : 3* |
| Control, unsprayed | | | | | | | | |
| NL | — | 21·9 | 6·8 | — | 4·4 | 5·0 | 23·9 | 38·0 |
| MGDG | 9·1 | 23·0 | — | 5·1 | 4·3 | 5·4 | 16·4 | 36·7 |
| DGDG | 7·5 | 42·9 | 1·0 | — | 8·4 | 6·8 | 11·7 | 21·7 |
| PL | — | 14·8 | — | — | 4·3 | 28·4 | 36·4 | 16·1 |
| Control, sprayed | | | | | | | | |
| NL | — | 18·8 | 8·8 | — | 4·0 | 4·9 | 16·1 | 47·4 |
| MGDG | 38·0 | 41·0 | — | — | 8·9 | 12·1 | — | — |
| DGDG | 6·0 | 34·8 | — | — | 5·9 | 6·1 | 12·3 | 34·9 |
| PL | — | 37·9 | — | — | 3·8 | 7·6 | 11·9 | 38·8 |
| Glyphosate-tolerant regenerant, unsprayed | | | | | | | | |
| NL | tr | 25·1 | 3·6 | — | 3·9 | 4·5 | 24·4 | 38·5 |
| MGDG | 31·7 | 56·3 | — | — | 8·7 | 3·3 | — | — |
| DGDG | 22·2 | 56·9 | 1·5 | — | 10·6 | 6·0 | 2·8 | — |
| PL | 5·6 | 57·2 | 2·2 | — | 6·9 | 6·0 | 12·9 | 9·2 |
| Glyphosate-tolerant regenerant, sprayed | | | | | | | | |
| NL | 2·5 | 17·2 | 5·2 | 12·9 | 3·3 | 4·4 | 13·7 | 40·8 |
| MGDG | 6·2 | 13·7 | — | 8·4 | 2·9 | 3·5 | 8·4 | 56·9 |
| DGDG | 2·3 | 17·6 | — | — | 6·1 | 4·7 | 11·5 | 57·8 |
| PL | — | 36·0 | 4·4 | — | 4·0 | 7·7 | 17·3 | 30·6 |

[a] See footnote to Table 3.

selected from calluses surviving on glyphosate. In the regenerants from *Zlatna arda*, the changes were mainly in GL concentrations—the level of MGDG decreased, while the level of DGDG increased. The total amount of GL remained the same and this is an indication that there were no changes in the overall rate of biosynthesis of GL, but only an increase of the galactosylation of MGDG to DGDG. The reduction of the amounts of MGDG would be expected to decrease substantially the cell membrane permeability. This decrease is much

**TABLE 5**
Influence of Glyphosate on the Sterol Composition in Leaves of Two Tobacco Cultivars

| | (% w/w) | | | | | |
|---|---|---|---|---|---|---|
| *Sample* | *iso-fucosterol* | *sitosterol* | *campesterol* | *stigmasterol* | *cholesterol* | $\Delta^{22}$ *cholesterol* |
| *Zlatna arda* | | | | | | |
| Control, unsprayed | tr | 23 | 19 | 46 | 7 | 1 |
| Control, sprayed | 1 | 16 | 17 | 55 | 7 | tr |
| Glyphosate-tolerant regenerant, unsprayed | tr | 21 | 21 | 44 | 4 | tr |
| Glyphosate-tolerant regenerant, sprayed | 3 | 22 | 26 | 37 | 5 | tr |
| *Nevrokop* $A_{24}$ | | | | | | |
| Control, unsprayed | 1 | 21 | 19 | 44 | 5 | tr |
| Control, sprayed | tr | 22 | 22 | 41 | 5 | 1 |
| Glyphosate-tolerant regenerant, unsprayed | tr | 18 | 21 | 42 | 5 | 3 |
| Glyphosate-tolerant regenerant, sprayed | 3 | 14 | 17 | 55 | 5 | 4 |

bigger than the decrease obtained when wild-type plants were treated with glyphosate (see above).

Surprisingly, in the *Nevrokop* $A_{24}$ regenerant, there were no GL changes. A large increase in NL concentrations and a decrease of the amounts of PL were observed. Evidently, the eventual adaptation of *Nevrokop* $A_{24}$ callus towards glyphosate was accompanied by changes of PL concentrations and not by GL changes, as in *Zl.arda*.

The changes in the sterol composition in regenerants were insignificant (Table 5).

We could expect substantial differences in the lipid and sterol composition after spraying of the regenerants with glyphosate. It could be expected that the regenerants would be more resistant to glyphosate than controls. Surprisingly, the results obtained showed that MGDG concentrations in treated regenerants were higher than in controls, which means lower control on ion permeability and probably lower adaptation toward the herbicide (Tables 1 and 2).

The treatment with glyphosate of *Zl.arda* regenerant led to increase of campesterol and decrease of stigmasterol concentrations. The same treatment of *Nevrokop* $A_{24}$ regenerant resulted in a significant increase in stigmasterol.

In our study the plants surviving more than 60 days (about 10 times longer than the control) after spraying with glyphosate were considered as tolerant. Insofar as we did not succeed in obtaining seeds from them, we are still not able to state that glyphosate-resistant clones were obtained.

## 4 CONCLUSION

The data obtained show unambiguously that the lipid and sterol composition of tobacco plants significantly changes after treatment with glyphosate. Most of the changes would be expected to decrease the cell membrane permeability.

Spraying with glyphosate of regenerants from calluses selected on herbicide-containing media also led to some changes in the lipid composition. It is evident, however, that these changes were not enough to protect the plants from the phytotoxic effect at the main targets of glyphosate action.

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